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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)		
Given name (first and middle [if any])	Family Name or Surname	Residence (city and either State or Foreign Country)
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Additional inventors are being named on the ___ separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

TUMOR-SPECIFIC EXPRESSION OF REPORTER GENES

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ENCLOSED APPLICATION PARTS (*check all that apply*)

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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

SIGNATURE _____ Date August 28, 2003

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This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Heyer et al.

For : TUMOR-SPECIFIC EXPRESSION OF REPORTER GENES

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1. Provisional Application Cover Sheet (in duplicate);

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Claire J. Sullivan Goodman

TUMOR-SPECIFIC EXPRESSION OF REPORTER GENES

FIELD OF THE INVENTION

The invention relates to compositions and methods for detecting tumors in an animal.

BACKGROUND OF THE INVENTION

Tumors develop from normal cells in a tissue by acquiring mutations in their genome that help to overcome the growth restriction in the tissue. Imaging developing tumors is a challenging task due to the fact that the initial tumor mass is very small compared to the normal tissue mass. The current method for visualizing the development of tumors uses tissue-specific expression of a reporter gene (e.g., Vooijs et al., Cancer Res. 62(6):1862-7 (2002)). Because all cells in the tissue express the reporter, only larger tumor masses can be seen in imaging. Early stage, small tumors will go undetected.

SUMMARY OF THE INVENTION

This invention provides a reporter gene that is preferentially expressed in a tumor tissue as compared to the normal, originating tissue. This reporter gene encodes a detectable product and is linked operably to a transcriptional regulatory element that is up-regulated by a tumor-induced transcription factor, e.g., a transcription factor that is specifically or preferentially active in a tumor tissue. The

reporter gene product can be a light-emitting substance such as a fluorescent protein (e.g., a green fluorescent protein) or a luminescent protein (e.g., a luciferase).

This invention allows highly sensitive detection of small, developing tumors that are otherwise undetected by existing methods. Additionally, the invention offers the advantage of decreasing the number of animals and the tumor load in each animal needed to screen potential therapeutic compounds.

Other features and advantages of the invention will be apparent from the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

Tumor development often affects key signaling pathways in a tumor cell, resulting in increased activities of one or more transcription factors controlled by these pathways in the tumor cell as compared to a normal cell. This invention features a reporter gene placed under the control of a transcriptional regulatory element that is up-regulated by such a tumor-induced transcription factor. A tumor cell expresses the reporter gene at a higher level than its normal counterpart. Thus, the reporter gene can be used to detect tumors in live animals at any stage of tumor development, e.g., during early stages of tumorigenesis, even when the tumor is too small to be detected by convention methods.

Methods of the invention can be used to study the development (e.g., initiation, progression, maintenance, metastasis, regression, minimal residual disease, recurrence, and any other developmental stages) of cancer in an objective, real time, quantitative and noninvasive manner. This approach to tumor imaging is a significant advance for rapid and dynamic screening as well as validation of experimental therapeutic agents.

I. TUMOR-SPECIFIC REPORTER GENES

The reporter gene of this invention is operably linked to a transcriptional regulatory element, e.g., a promoter or enhancer, that is up-regulated by a tumor-induced transcription factor. As such, the reporter gene will have basal expression levels in a normal tissue and higher expression levels in a tumor tissue. A

"transcription factor," as described herein, refers to a protein which is involved in the transcription of genes. "Tumor-induced transcription factors" are those whose expression or activity is increased during tumor development.

An example of tumor-induced transcription factors is β-catenin. β-catenin regulates cell proliferation and differentiation in the mucosal membrane where gastrointestinal tumors typically arise. β-catenin is normally sequestered in the cytoplasm by the Adenomatous Polyposis Coli (APC) protein. APC is a gastrointestinal tumor suppressor, the inactivation of which (e.g., due to deletion of the APC gene) is a hallmark of many gastrointestinal tumors, including colon cancer. When APC is inactivated, β-catenin relocates to the nucleus and forms a transcription factor complex with T Cell Factor 4 (TCF4) and other transcription factors. The transcription factor complex binds to promoters that have the corresponding binding sites, resulting in expression of genes controlled by these promoters. Examples of such genes are those encoding c-myc, CD44, BMP4, CLAUDIN I, Cyclin D1, FRA1, NrCAM, PKD1, Survivin, and Ephrin B (Giles et al., Biochimica et Biophysica Acta 1653:1-24 (2003); Kim et al., Lancet 362:205-9 (2003)). Thus, expression of a reporter gene operably linked to a promoter controlled by the β-catenin transcription factor complex, such as the promoter of one of the aforementioned genes or an artificial Top/Fop promoter (see below), is indicative of tumor formation in the gastrointestinal tissue.

β-catenin is also activated in hepatocarcinoma caused by loss of Axin activity. Other examples of tumor-induced transcription factors are Forkhead, which is activated in prostate cancer caused by loss of PTEN activity; and Smad-2 and Smad-3, which are activated in pancreatic cancer caused by loss of Smad-4 or DPC-4 activity. A reporter gene linked to a transcriptional regulatory element up-regulated by one of these transcription factors is useful in monitoring tumors in the liver, prostate gland, and pancreas, respectively.

When expressed in a cell, a reporter gene of this invention produces a detectable phenotypic change in the cell. The reporter gene may encode a product whose activity is not normally found in the organism of interest and thus may be easily

assayed, or encode a product that is naturally found in the organism of interest but not naturally found in the tissue that gives rise to the tumor. Useful reporter genes include those encoding enzymes, enzymatic substrates, luminescent proteins, and fluorescent proteins, such as luciferase, β-galactosidase, alkaline phosphotase, chloramphenicol acetyltransferase, green-fluorescent protein (GFP), and variants of GFP. Assays for reporter genes are well known in the art. Reporter genes, assay kits and other materials are available commercially from suppliers such as Promega Corp. (Madison, WI) and GIBCO BRL (Gaithersburg, MD).

In some embodiments, a gene encoding luciferase or an equivalent thereof is used. Members of the luciferase family have been identified in a variety of prokaryotic and eukaryotic organisms. Luciferase and other enzymes involved in the prokaryotic luminescence systems, as well as the corresponding lux genes, have been isolated from marine bacteria in the *Vibrio* and *Photobacterium* genera and from terrestrial bacteria in the *Xenorhabdus* genus. Eukaryotic organisms (e.g., firefly) can also be used to obtain luciferases.

In other embodiments, a gene encoding GFP is used. GFP is a fluorescent protein isolated from coelenterates such as the Pacific jellyfish *Aequoria victoria*. There are many useful variants of GFP that can also be used in this invention. They include, without limitation, enhanced GFP (EGFP), yellow fluorescent protein, red fluorescent protein, blue fluorescent protein, etc. Constructs encoding these fluorescent proteins are available commercially (e.g., from Amersham).

The reporter gene of the invention can be inserted into a vector such as a viral vector (e.g., retroviral vectors such as murine leukemia viral vectors, adenovirus vectors, herpes virus vectors, and lentiviral vectors), a plasmid vector, or an artificial chromosome (e.g., a BAC, a PAC, a YAC or a MAC). These vectors can be introduced into a host cell (e.g., an oocyte, a embryonic or tissue-specific stem cell, or a differentiated cell) via infection, microinjection, transfection, transposome/ liposome fusion, and the like.

II. USE OF TUMOR-SPECIFIC REPORTER GENES

The tumor-specific reporter genes of this invention can be used to detect tumors *in vivo*. The reporter gene is introduced into a pre-malignant cell (or an ancestor thereof) in an animal. Once the cell becomes malignant, the expression or activity of a tumor-induced transcription factor is increased, which in turn up-regulates the transcriptional regulatory element linked operably to the reporter gene. Expression of the reporter gene thus enables one to detect tumors *in vivo*, even when the tumors are too small to be detected by convention methods, e.g., physical examination.

Tumors in the animal can either arise spontaneously or be induced artificially *de novo*. Tumorigenesis can be induced by methods well known in the art, for example, via exposure to carcinogens or exposure to UV or gamma irradiation. Alternatively, the animal may contain cells whose genomes comprise a genetic mutation that renders them more susceptible to cancer than they would otherwise be.

In some embodiments, such a genetic mutation may be an introduced oncogene under inducible transcriptional control. Expression of the oncogene can be induced by, e.g., a Cre-Lox system and any of the inducible transcription systems for RNA polymerase II (e.g., the tetracycline transactivator systems, reverse tetracycline transactivator systems, ecdysone systems, methallothionine systems, LacO/IPTG systems, and TetO/tetracycline systems). See, e.g., WO 01/09308. Inducible transcription systems for RNA polymerases I and III can also be used with or without modifications.

A cancer-prone genetic mutations may also be disabling (e.g., null, conditionally null, or dominant negative) mutations in a tumor suppressor gene (e.g., INK4a, P53, APC, PTEN, Rb (Jacks et al., Nature 359:295-300 (1992), DPC4, KLF6, GSTP1, ELAC2/HPC2, or NKX3.1), disabling mutations in a DNA repair gene (e.g., MSH2, MSH6, PMS2, Ku70, Ku80, DNA/PK, ATR, ATM, XRCC4, or MLH1), and activating mutations in an endogenous proto-oncogene (e.g., *myc* and *ras*). These mutations can be introduced into the genome of a host cell by well established homologous recombination technologies (e.g., gene knock out or knock in).

Disabling mutations of a tumor suppressor gene or a DNA repair gene may be RNA interference (RNAi) constructs introduced into the host genome to inhibit expression of this gene. RNAi is a sequence-specific posttranscriptional gene silencing mechanism triggered by double-stranded RNA (dsRNA). It causes degradation of mRNAs homologous in sequence to the dsRNA. See, e.g., Elbashir et al., Methods 26:199-213 (2002); McManus and Sharp, Nature Reviews 3:737-747 (2002); Hannon, Nature 418:244-251 (2002); Brummelkamp et al., Science 296:550-553 (2002); Tuschl, Nature Biotechnology 20:446-448 (2002); U.S. Patent 6,506,559; U.S. Application US2002/0086356 A1; WO 99/32619; WO 01/36646; and WO 01/68836. With the use of RNAi constructs, one can further control tumorigenesis in the animal by inducibly expressing RNAi molecules that interrupt the function of the tumor suppressor and/or DNA repair activity.

Preferably, the reporter gene and the cancer-prone genetic mutation (or mutations) are contained within the same cells so that the reporter gene indicates only tumor formation caused by the genetic mutation. This can be achieved by, e.g., introducing into a host cell sequentially or concurrently a vector containing the reporter gene and a vector containing the genetic elements for causing the genetic mutation. This can also be achieved that by introducing into a host cell a vector that contains both the reporter gene and those genetic elements. In some embodiments, the cell contains more than one cancer-prone genetic mutation. For example, the cell may contain an inducible oncogene and a null mutation in an endogenous tumor suppressor gene.

The vectors may be administered into host cells via a variety of methods, including but not limited to, liposome/transposome fusion, routine nucleic acid transfection methods such as electroporation, calcium phosphate precipitation, and microinjection, and infection by viral vectors. The vectors may be introduced into host cells in an animal *in vivo* or *ex vivo*. For instance, the vectors may be administered to any tissue in an animal, including without limitation: dermal, brain, heart, lung, kidney, colon, gastrointestinal, prostate, ovarian, breast, liver or bone tissue. The vectors can also be introduced into tissue-specific stem cells (including

progenitor cells) *ex vivo*, which are then implanted into a desired tissue in an animal. The vectors can also be introduced into embryonic stem cells which are used to generate a transgenic or chimeric (including mosaic) animal. The vectors can also be injected into a fertilized oocyte which is then developed into a transgenic animal.

Alternatively, the reporter gene is introduced into a transgenic animal containing the cancer-prone genetic mutation or into cells in a chimeric animal that contain the cancer-prone genetic mutation. Or, an animal containing the reporter gene can be bred with an animal containing the genetic mutation to produce a progeny which contains both the reporter gene and the genetic mutation.

An increase in expression of the reporter gene can be detected by, e.g., measuring the intensity of light emission from a light-emitting product encoded by the reporter gene. Light emission may be measured by standard techniques, e.g., through use of a luminometer, photometer, camera or other photon detecting device.

III. EXAMPLES

This example describes the use of a reporter gene to study colon cancer in mice via *in vivo* bioluminescence imaging of luciferase expression. The example is intended to illustrate the methods and materials of the present invention. Suitable modifications and adaptations of the described conditions and parameters normally encountered in the art which are obvious to those skilled in the art are within the spirit and scope of the present invention. This examples is not to be construed as limiting the scope of the invention in any way.

The mouse used here contains gastrointestinal cells whose APC gene can be conditionally knockout. For example, these cells contain an inducible RNAi construct that specifically inhibits the expression of the APC gene; alternatively, a pair of LoxP sites have been inserted into the endogenous APC gene of these cells, and upon expression of a Cre recombinase in these cells, the APC gene is knocked out. These cells contain also a reporter gene construct comprising a luciferase-coding sequence linked operably to an artificial Top/Fop promoter (Staal et al., International Immunology 11:312-7 (1999)). Colon cancer is induced in the mouse by inactivating the APC gene in the gastrointestinal tissue. Upon formation of colon cancer, the

reporter construct starts to express luciferase, which can be detected by well known methods (Contag and Bachmann, Annual Review of Biomed. Eng. 4:235-60 (2002)). Light emission will be observed at a basal level in normal gastrointestinal tissues that have not undergone tumorigenesis, and at an increased level in those that have undergone tumorigenesis.

This mouse model permits longitudinal monitoring of tumor onset, progression, and response to therapy and may be used effectively for testing cancer prevention and treatment strategies based on therapeutics that specifically target the APC pathway.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. All publications and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting. Throughout this specification and claims, the word "comprise," or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Abstract

This invention features a reporter gene operably linked to a transcriptional control element that is up-regulated by a tumor-induced transcription factor. This reporter gene can be used to detect tumor formation *in vivo* in an animal.

5

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